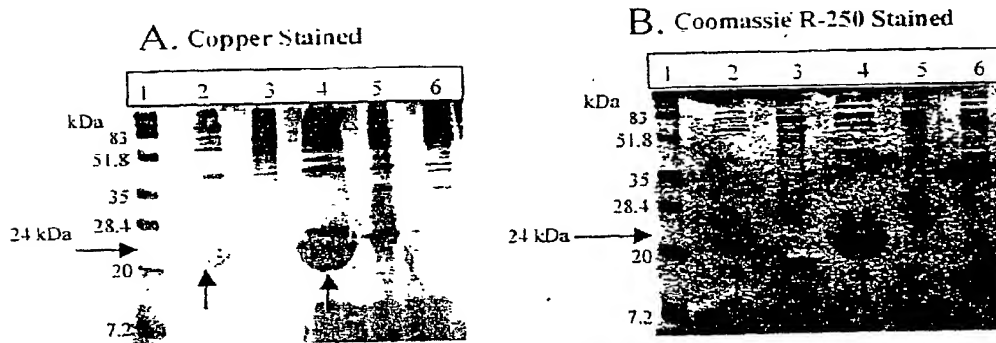


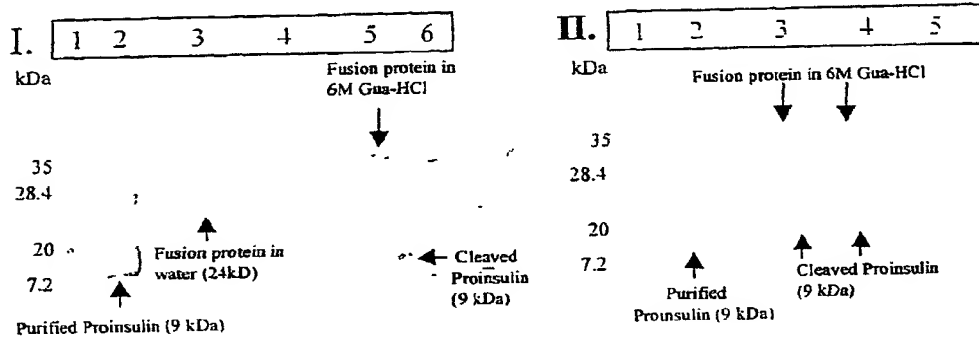
**Figure 1: Biopolymer-Proinsulin Fusion Protein Expression**

PAGE: 15% Glycine large gel

**A. Copper Stained:** Gel rinsed in water for 10 min, stained with 0.3M  $\text{CuCl}_2$  for 5min, and rinsed in water for 3min.

**B. Coomassie R-250 Stained:** The same gel was first rinsed for 20min in water and then stained for 1hr, and destained overnight.

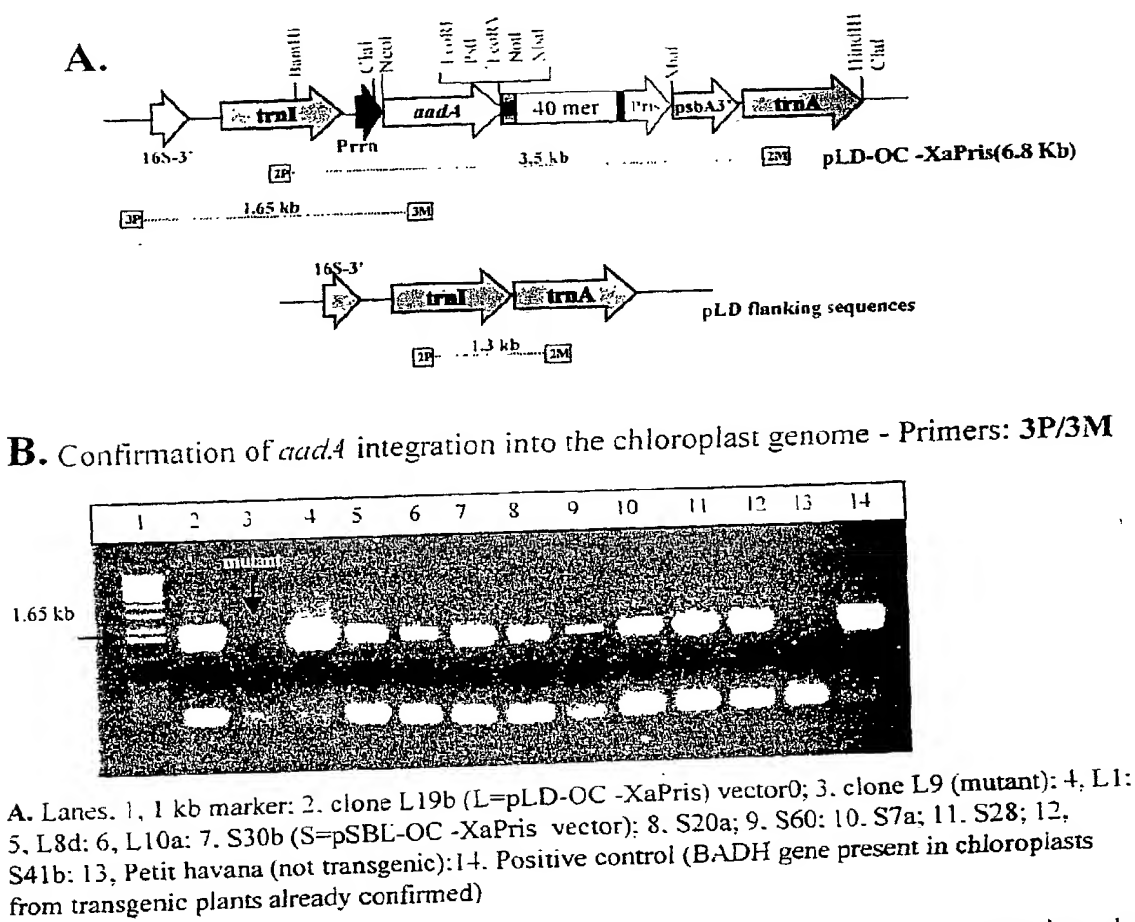
**A. and B. Lanes.** 1. Prestained Marker (BioRad); 2. Sonic extract of pSBL-OC-XaPris; 3. reverse orientation of fusion protein of pSBL-OC-XaPris; 4. Sonic extract of pLD-OC-XaPris; 5. inverse orientation of pLD-OC-XaPris; 6. Sonic extract of *E. coli* strain XL-1 Blue containing no plasmid.

**C. Western Blot of Biopolymer-Proinsulin Fusion Protein**

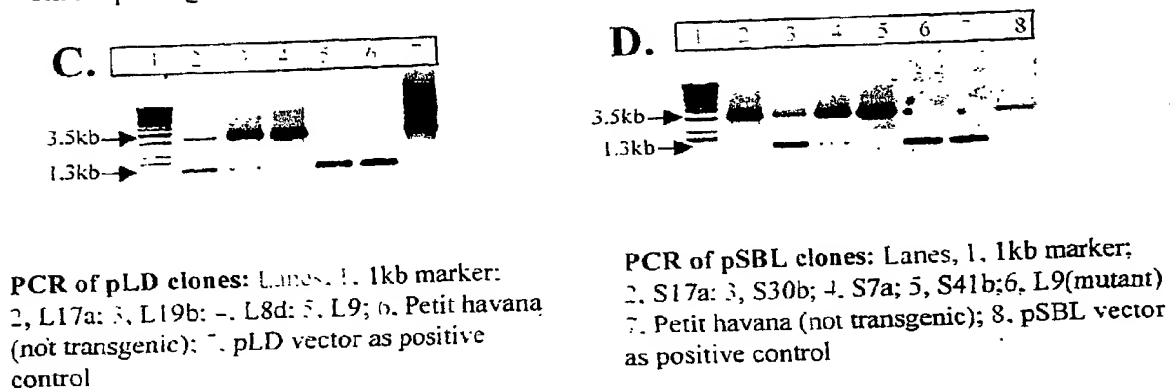
**I. Lanes.** 1, BioRad Prestained Marker; 2, 3ug of Purified Human Proinsulin; 3. 5ug of pSBL-OC-XaPris (sonication and purification of biopolymer twice); 4. Negative control, XL-1 Blue *E. coli*; 5, Sonic extract pSBL expressing cells (6M Guanidine Hydrochloride Phosphate Buffer, pH 7.0); 6, Sonic extract of XL-1 Blue *E. coli* with no pSBL.

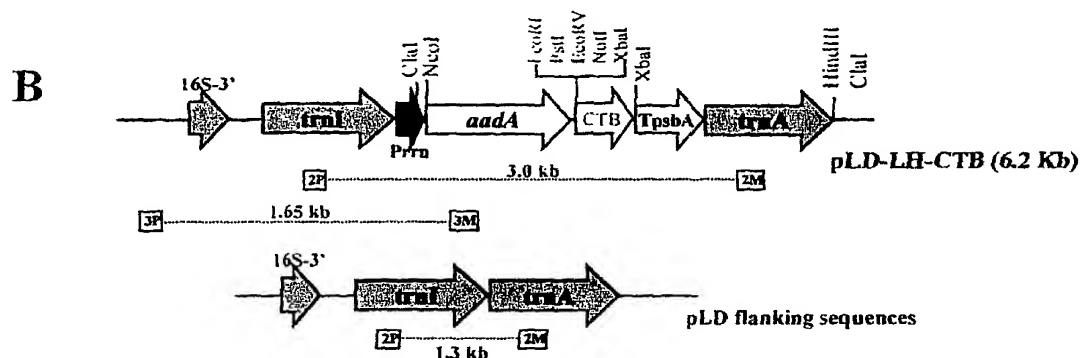
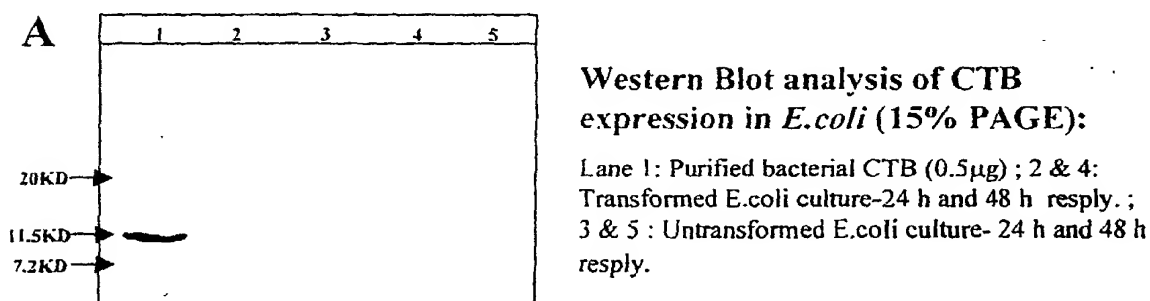
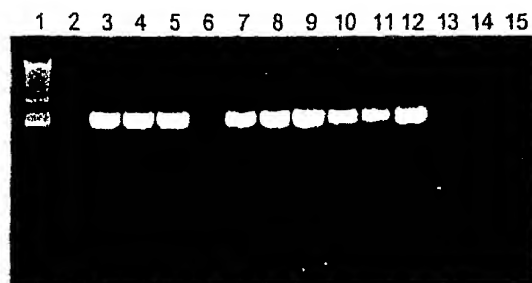
**II. Lanes.** 1. BioRad Prestained Marker; 2, 5ug of Purified Human Proinsulin; 3, Sonic extract of pSBL-OC-XaPris expressing cells (6M Guanidine Hydrochloride Phosphate Buffer, pH 7.0); 4. Sonic extract of pLD-OC-XaPris expressing cells (Gua-HCl); 5, Sonic extract of XL-1 Blue *E. coli* with no plasmid.

## Figure 2: Confirmation of Chloroplast Integration by PCR of Polymer-Proinsulin Fusion Gene

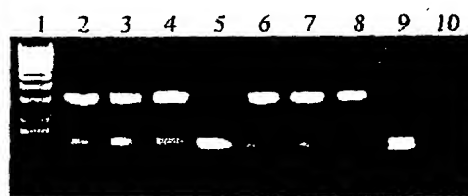


Confirmation of integration of *aadA* and biopolymer-proinsulin fusion genes into the chloroplast genome - Primers: 2P/2M



**Figure 3 : CTB Gene Expression and Chloroplast Integration****C. PCR confirmation of *aadA* gene integration into chloroplast genome -3P/3M primers**

**PCR of clones of 1st. round of selection :**  
 Lane 1: 1 Kb marker ; 2 - 12 : Plant total DNA from spec. clones 1-11 (Note: Lanes 2 & 6 are mutants); 13: Untransformed plant; 14: pLD-LH-CTB vector ; 15: No DNA template.

**D. PCR confirmation of integration of *aadA* and CTB gene into chloroplast genome - 2P/2M primers**

**PCR of clones of 2nd. round of selection :**  
 Lane 1: 1 Kb marker; 2 - 7 : Plant total DNA from spec. clones 1- 6 (Note: Lane 5 is a mutant); 8: pLD-LH-CTB vector; 9: Untransformed plant ; 10 : No DNA template.

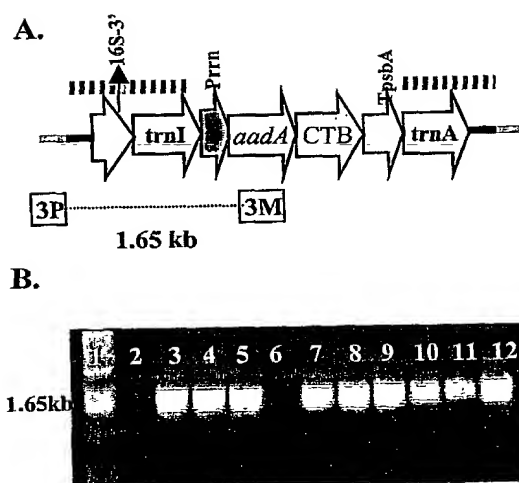


Figure 1



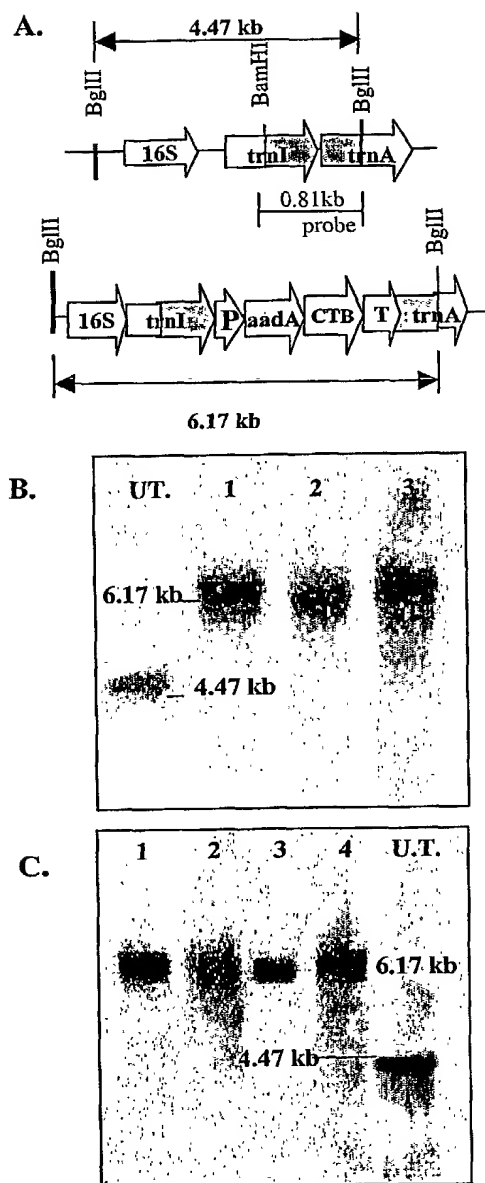


Figure 3

WO 01/72959

7/20

PCT/US01/06288

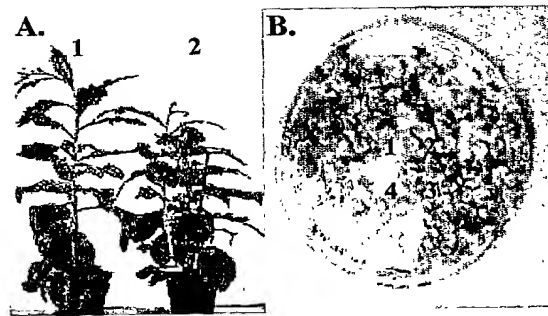


Figure 4





• Expression of bacterial operon in transgenic chloroplasts.

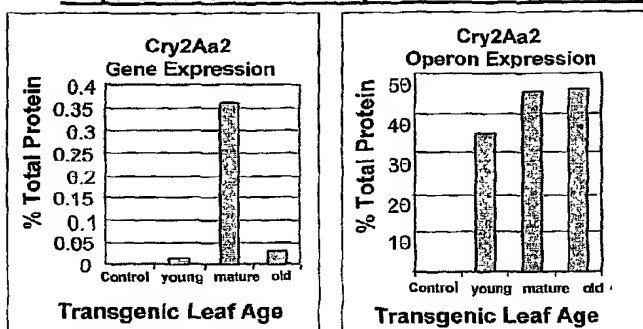


Figure 1: Cry2A protein concentration determined by ELISA in transgenic leaves. Note 100-fold increase in protein accumulation in the presence of the putative chaperonin, ORF2.

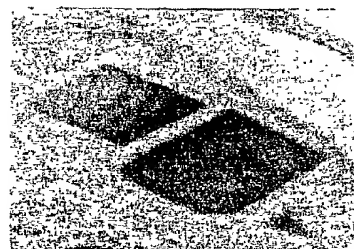


Figure 2: Immunogold labeled electron microscopy of mature transgenic leaf. Cry2Aa2 crystals in a transgenic chloroplast expressing the cry2A operon.

• Expression of a small (22aa) peptide in transgenic chloroplasts.

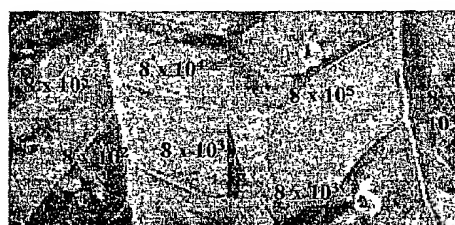


Figure 3: Leaves were infected with 10  $\mu$ l of  $8 \times 10^5$ ,  $8 \times 10^4$ ,  $8 \times 10^3$  and  $8 \times 10^2$  cells of *P. syringae*. Photos were taken 5 days after inoculation. 1-2  $\mu$ g of antimicrobial peptide (AMP) is required to kill 1000 bacterial cells. Local concentration at the site of infection is estimated to be 200-800  $\mu$ g AMP.

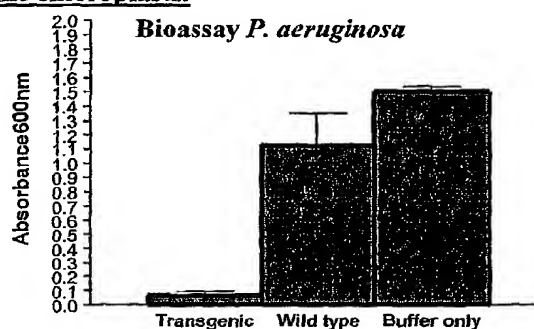


Figure 4: Total plant protein was mixed with 5  $\mu$ l of mid-log phase bacteria from overnight culture, incubated for 2 hours at 25°C at 125rpm and grown in LB broth overnight. Based on minimum inhibitory concentration of 1-2  $\mu$ g AMP/1000 bacterial cells, the expression level was calculated to be 21.5-43% of the total soluble protein.

• Expression of Oligomeric form (disulfide bonded) CTB in transgenic chloroplasts.

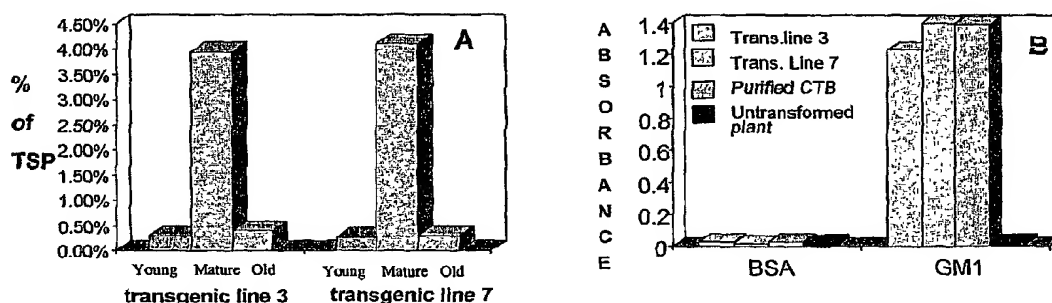
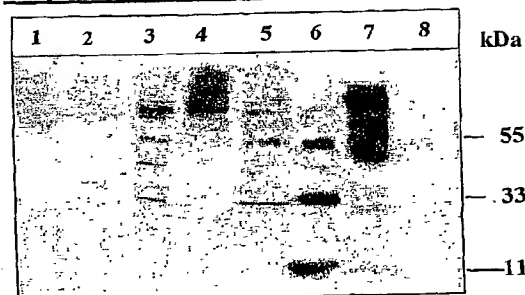


Figure 5: A) CTB ELISA quantification is shown as a percentage of the total soluble plant protein. Total soluble plant protein from young, mature and old leaves of transgenic lines 3 and 7 was quantified. B) CTB-GM1 Ganglioside binding ELISA assays: Plates coated first with GM1 gangliosides and BSA were plated with total soluble plant protein from lines 3 and 7, untransformed plant total soluble protein and purified bacterial CTB. The absorbance or the GM1 ganglioside-CTB antibody complex was measured.

10/20

### • Expression of CTB oligomers.



**Figure 6:** 12% reducing PAGE. Chemiluminescent detection with rabbit anti-cholera serum (1<sup>0</sup>) and AP labeled mouse anti-rabbit IgG (2<sup>0</sup>) antibodies. Untransformed, boiled (1) and unboiled (2); Transformed, boiled (3&5) and unboiled (4); Purified CTB boiled (6) and unboiled (7); Marker (8).

### • Marker-free chloroplast transgenic plants.

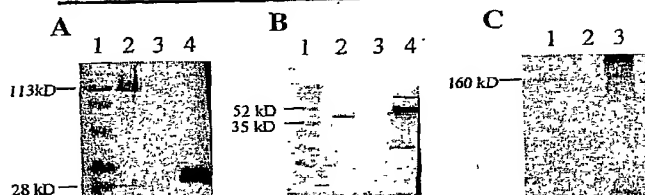
Selectable marker	Plate No.	Total no. of leaf discs	No of responding leaf discs	Total no of shoots/ plate
BADH	1	3	3	43
	2	6	4	23
	3	11	9	33
	4	7	6	19
	5	6	4	16
	6	9	7	18
Spectinomycin	1	5	0	0
	2	5	0	0
	3	5	3	3
	4	5	2	2
	5	5	0	0
	6	5	1	1
	7	5	1	2
	8	5	1	2
	9	5	0	0
	10	5	0	0
Control		5	0	0

**Table 1:** Comparison of Spectinomycin and Betaine aldehyde as the selectable marker for the first round of selection.

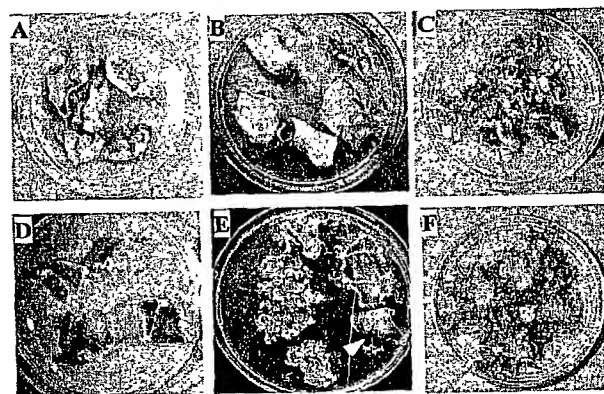
### • Codon composition and expression levels.

Open reading Frame	% TSP	% A+T	% psbA	% cp tRNA
Plastid miniprinsulin	?	66	100	62
CTB	4.1	66	47	34
Cry2A operon	47	65	37	37
Plastid proinsulin	?	64	100	49
Antimicrobial peptide	21	63	35	35
Guys light chain	<1%	49	31	44
Optimized biopolymer	?	47	100	40
Guys heavy chain	<1%	40	25	44
Human proinsulin	?	38	26	44

### • Expression & assembly of disulfide bonded Guy's 13 monoclonal antibody.

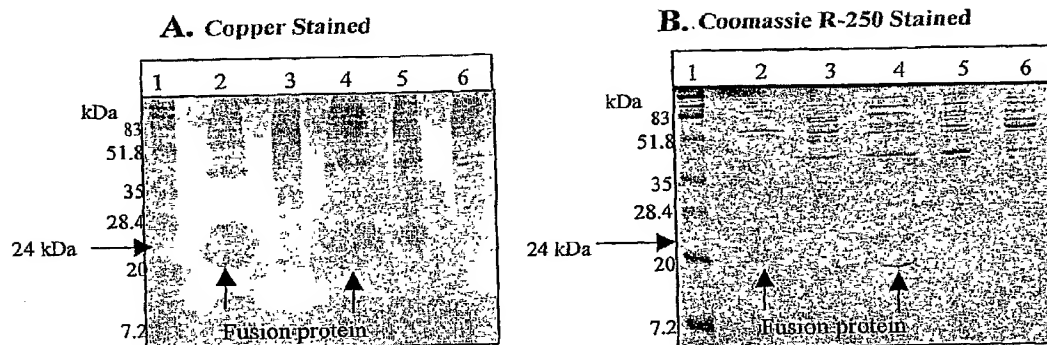


**Figure 7:** A, B) reducing gels. 1: markers, 2: Transgenic extract showing expression of light (A) and heavy chain (B) in chloroplasts, 3: Untransformed, 4: Human IgA. C) non-reducing gel. 1: Transgenic extract showing assembly, 2: Untransformed, 3: Human IgA. Blots A & C were detected with AP conjugated goat anti-human kappa antibody. Blot B was detected with AP conjugated goat anti-human IgA antibody.

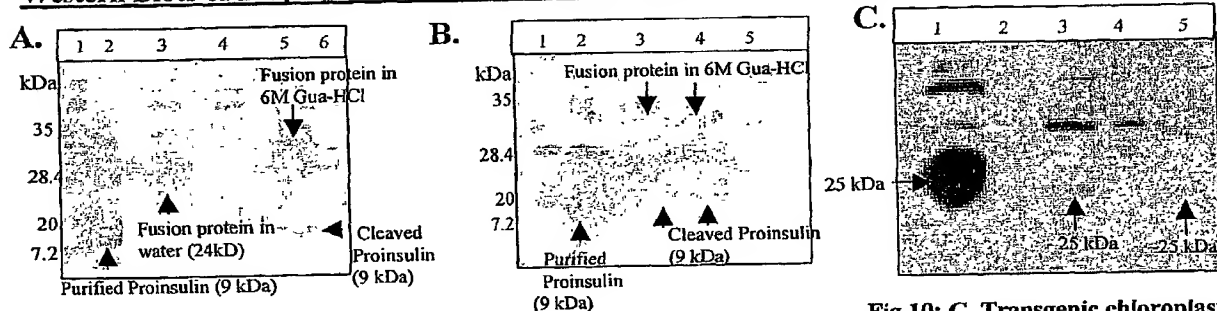


**Figure 8:** Comparison of betaine aldehyde and spectinomycin selection. A. *N. tabacum* Petit Havana control in RMOP medium containing spectinomycin after 45 days. B. Bombarded leaf discs selected on spectinomycin in RMOP medium after 45 days. C. Spectinomycin resistant clones cultured again (second round) to obtain homoplasmy. D. Petit Havana control in RMOP medium containing Betaine Aldehyde after 12 days of culture. E. Bombarded leaf discs selected on Betaine Aldehyde in RMOP medium after 12 days of culture; arrow indicates unbombarded leaf disc as control. Note that 23 shoots are formed on a disc selected on betaine aldehyde against 1-2 shoots per disc on spectinomycin. F. Betaine aldehyde resistant clones cultured again (second round) to obtain homoplasmy.

**Table 2 (Left):** Black indicates genes with unmodified native codon composition and their expression levels observed in transgenic chloroplasts, ranked by AT% in ascending order. Red indicates genes to be investigated. Kusnadi et al. (1997) suggest that a minimum of 1% TSP is adequate for commercial feasibility. See section d) for details of AT content, %psbA optimal codons and % of codons that match the cp tRNA pool. TSP: % total soluble protein

**Biopolymer-Proinsulin Fusion Protein Expression**

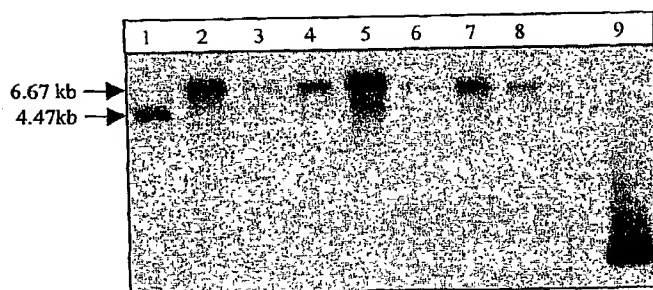
**Fig 9, A and B** Lanes: 1, Prestained Marker (BioRad); 2, Sonic extracts of pSBL-OC-XaPris; 3, reverse orientation of insert in pSBL-OC-XaPris; 4, pLD-OC-XaPris; 5, reverse orientation of pLD-OC-XaPris; 6, *E. coli* XL-1 Blue cells with no plasmid.

**Western Blots of Biopolymer-Proinsulin Fusion Protein After Single Step purification**

**Fig 10: A.** *E. coli* expression and cleavage Lanes: 1, BioRad Prestained Marker; 2, 3ug of Purified Human Proinsulin; 3, 5ug of pSBL-OC-XaPris; 4, Negative control, reverse orientation; 5, pSBL expressing cells (6M Guanidine Hydrochloride Phosphate Buffer, pH7.0); 6, XL-1 Blue *E. coli* with no pSBL.

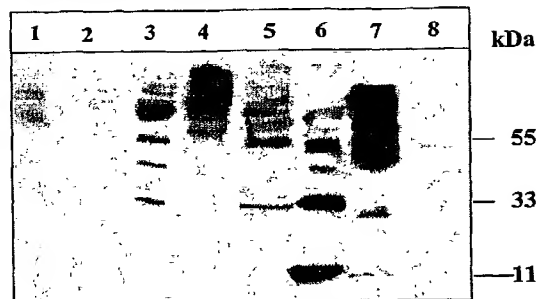
**Fig 10: B.** *E. coli* expression and cleavage Lanes: 1, BioRad Prestained Marker; 2, 5ug of Purified Human Proinsulin; 3, pSBL-OC-XaPris (6M Guanidine Hydrochloride Phosphate Buffer, pH7.0); 4, pLD-OC-XaPris; 5, XL-1 Blue *E. coli* with no plasmid.

**Fig 10: C.** Transgenic chloroplast expression Lanes: 1, Purified *E. coli* protein from pLD-OC-XaPris expression; 2, negative control (Petit Havana); 3-5, Chloroplast transgenic lines. Note dimer, tetramer and hexamer aggregates of polymer-insulin fusion protein

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**Fig11.** Biopolymer-proinsulin fusion gene integration into the chloroplast genome confirmed by Southern blot analysis. Lanes: 1, Petit Havana (negative control); 2-5, pLD-OC-XaPris clones T<sub>0</sub>; 6-8, pSBL-OC-XaPris clones T<sub>0</sub>; 9, probe(positive control). Homoplasmy is seen in most transgenic lines while a few transgenic lines show heteroplasmy.

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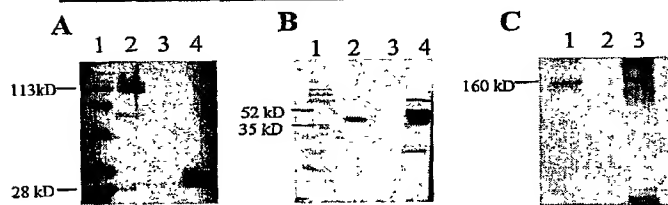
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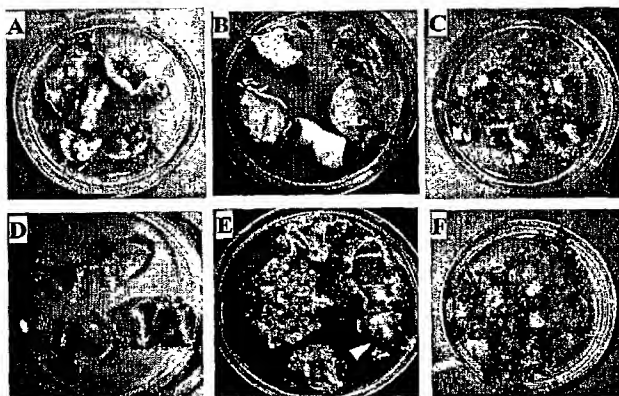
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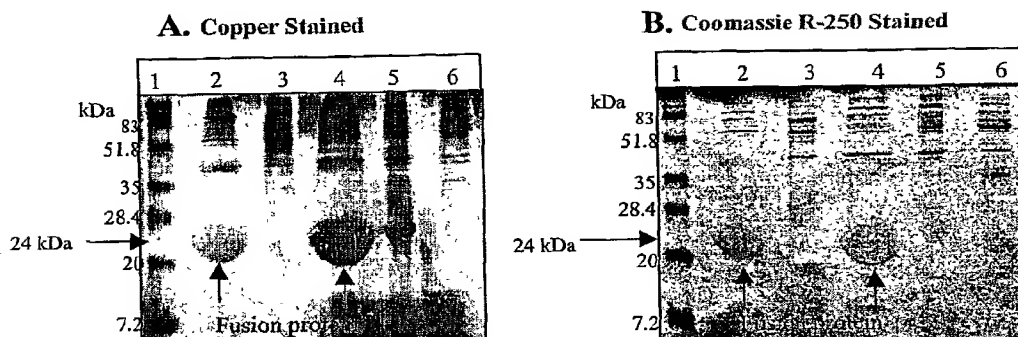
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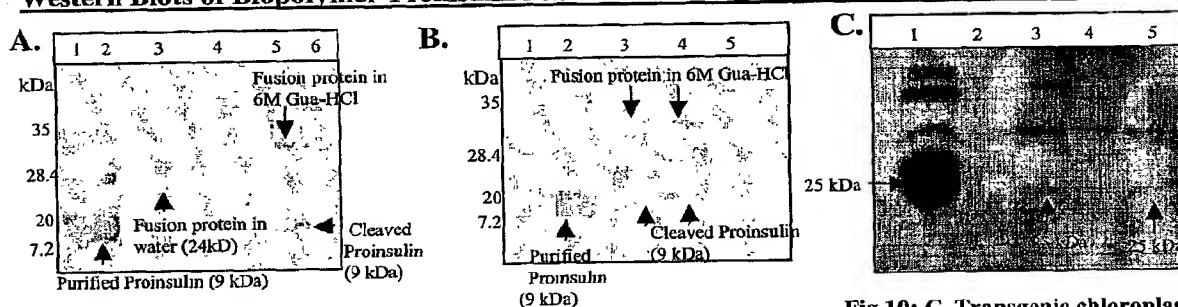
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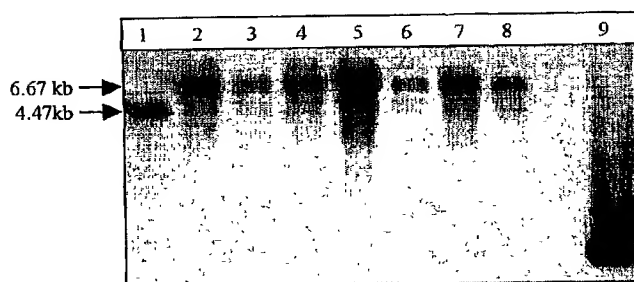


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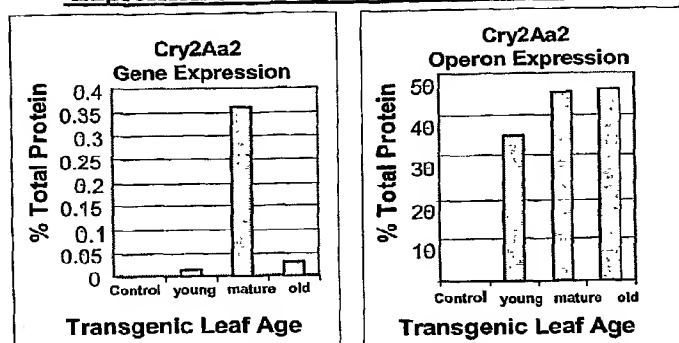
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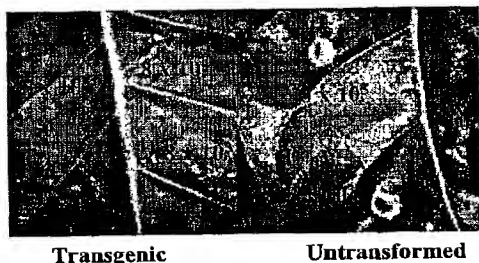


**Figure 1:** Cry2A protein concentration determined by ELISA in transgenic leaves. Note 100-fold increase in protein accumulation in the presence of the putative chaperonin, ORF2.

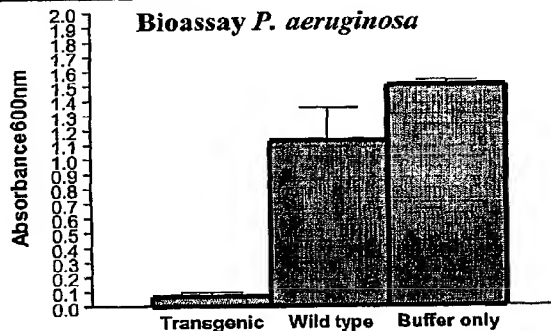


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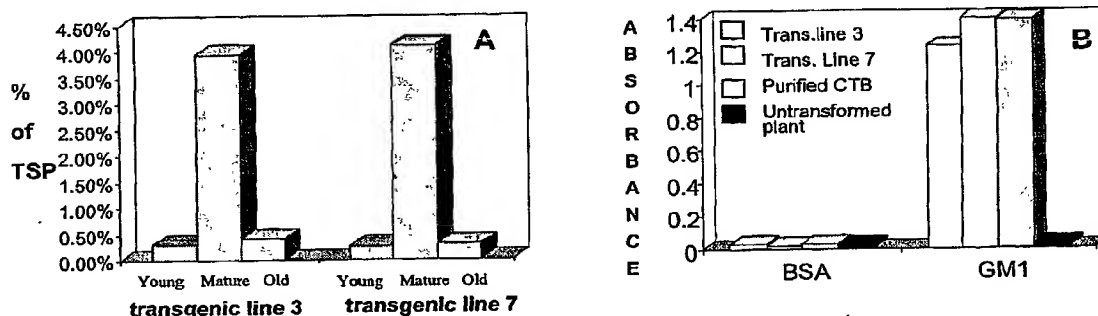


**Figure 3:** Leaves were infected with 10 µl of  $8 \times 10^5$ ,  $8 \times 10^4$ ,  $8 \times 10^3$  and  $8 \times 10^2$  cells of *P. syringae*. Photos were taken 5 days after inoculation. 1-2 µg of antimicrobial peptide (AMP) is required to kill 1000 bacterial cells. Local concentration at the site of infection is estimated to be 200-800µg AMP.

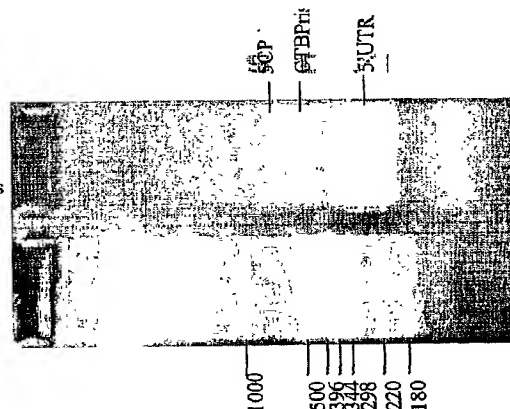


**Figure 4:** Total plant protein was mixed with 5µl of mid-log phase bacteria from overnight culture, incubated for 2 hours at 25°C at 125rpm and grown in LB broth overnight. Based on minimum inhibitory concentration of 1-2 µg AMP/1000 bacterial cells, the expression level was calculated to be 21.5-43% of the total soluble protein.

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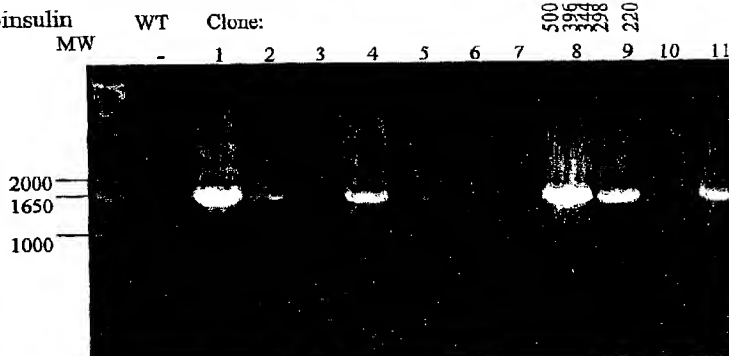


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**Figure 2** (above): SOEing of the 5'UTR to the CTB- human proinsulin sequence. 5CP is the PSbA 5'UTR and the Cholera Toxin B subunit (CTB) human proinsulin fusion

**Figure 4 (below): Recursive PCR to synthesize the chloroplast modified proinsulin (Ptpri)**



**Figure 5** (left): SOEing of the 5'UTR, CTB, and plasmid modified proinsulin, which results in the fusion of all three sequences denoted as **5CPTP**. The second lane show this



• **Expression of bacterial operon in transgenic chloroplasts.**

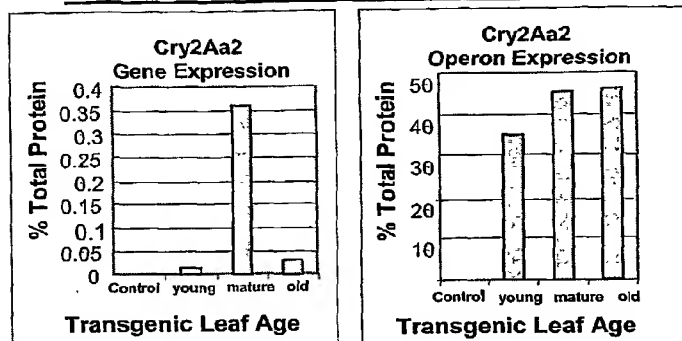


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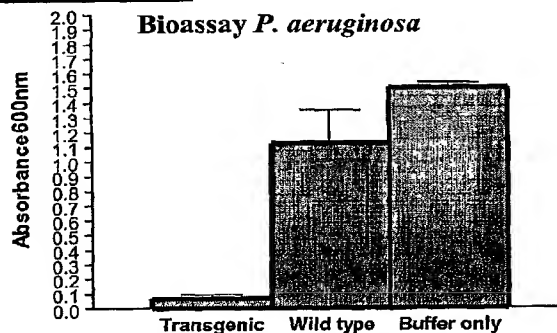


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• **Expression of Oligomeric form (disulfide bonded) CTB in transgenic chloroplasts.**

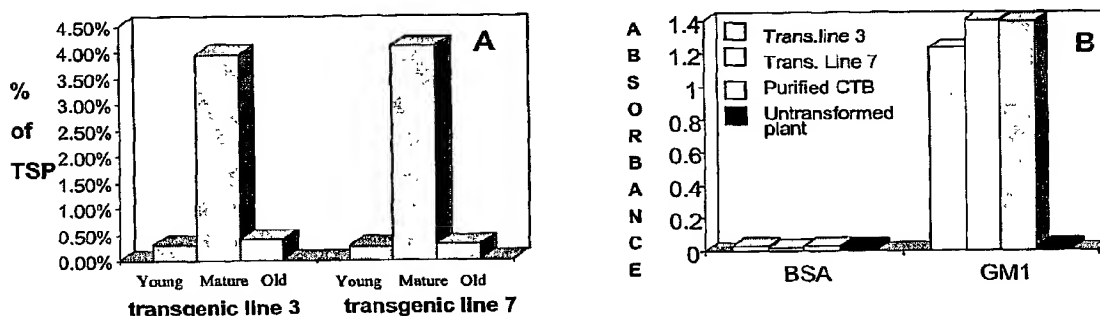
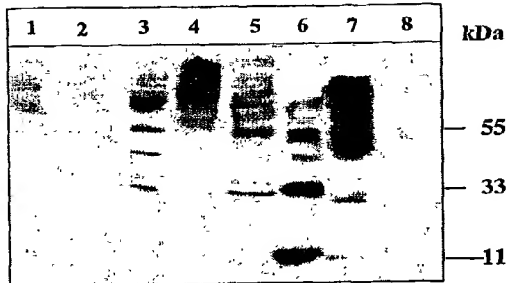


Figure 5: A) CTB ELISA quantification is shown as a percentage of the total soluble plant protein. Total soluble plant protein from young, mature and old leaves of transgenic lines 3 and 7 was quantified. B) CTB-GM1 Ganglioside binding ELISA assays: Plates coated first with GM1 gangliosides and BSA were plated with total soluble plant protein from lines 3 and 7, untransformed plant total soluble protein and purified bacterial CTB. The absorbance or the GM1 ganglioside-CTB antibody complex was measured.

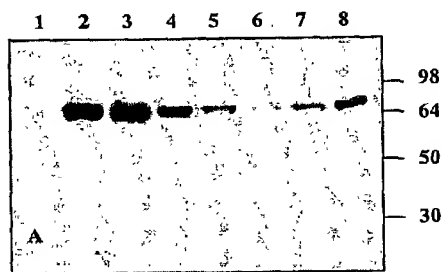


### • Expression of CTB oligomers.



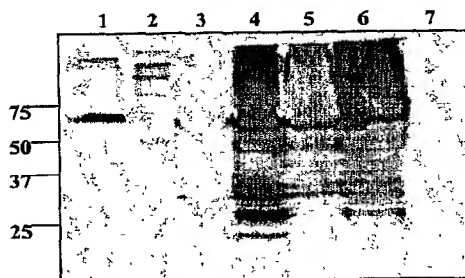
**Figure 6:** 12% reducing PAGE. Chemiluminescent detection with rabbit anti-cholera serum (1<sup>0</sup>) and AP labeled mouse anti-rabbit IgG (2<sup>0</sup>) antibodies. Untransformed, boiled (1) and unboiled (2); Transformed, boiled (3&5) and unboiled (4); Purified CTB boiled (6) and unboiled (7); Marker (8).

### • HSA Nuclear transformation of potato plants.



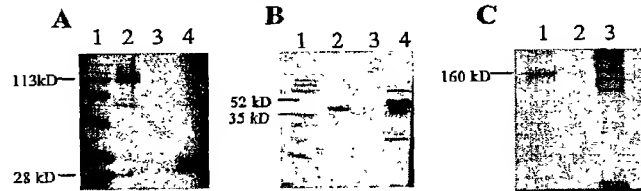
**Figure 8:** Western Blot of transgenic potato tubers, cv Désirée. 30 µg of tuber protein was loaded per lane and probed with anti-HSA antibody. 1: wild type; 2: 40 ng of pure HSA; 3-8: different transgenic lines, showing different levels of expression.

### • Expression of HSA by chloroplast vectors in *E. coli*.

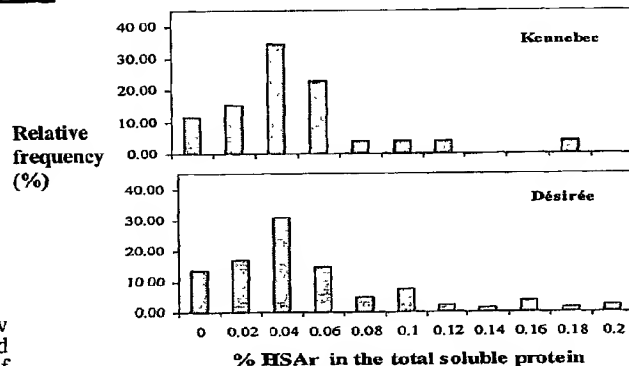


**Figure 10:** Western Blot of *E. coli* protein extracts. 1: 50 ng pure HSA; 2: molecular weight marker; 3: pLD-HSA (control without RBS); 4: PLD-5'UTR-HSA; 5: pLD-RBS-HSA; 6: pLD-ORF1+2-HSA; 7: *E. coli* without pLD vector.

### • Expression & assembly of disulfide bonded Guy's 13 monoclonal antibody.



**Figure 7:** A, B) reducing gels. 1: markers, 2: Transgenic extract showing expression of light (A) and heavy chain (B) in chloroplasts, 3: Untransformed, 4: Human IgA. C) non-reducing gel. 1: Transgenic extract showing assembly, 2: Untransformed, 3: Human IgA. Blots A & C were detected with AP conjugated goat anti-human kappa antibody. Blot B was detected with AP conjugated goat anti-human IgA antibody.



**Figure 9:** Frequency histogram including percentage Kennebec and Désirée transgenic plants expressing different HSA levels. Results are shown as the percentages of transgenic plants (vertical axis) that express a specific level of HSA of the total soluble protein (horizontal axis).

### • Codon composition and expression levels.

Open reading Frame	% TSP	% A+T	% psbA	% cp tRNA
CTB	4	66	47	34
Cry2A operon	47	65	37	37
Antimicrobial peptide	21-43	63	35	35
HSA	?	57	57	47
Interferon alpha	?	54	31	40
RUBISCOssTP	?	50	32	42
Guy's light chain	<1%	49	31	44
IGF-I	?	41	20	30
Guy's heavy chain	<1%	40	25	44

**Table 1:** Unmodified native codon composition and expression levels observed in transgenic chloroplasts. See section d) for details of AT content, %psbA optimal codons and % of codons that match the cp tRNA pool. TSP: % total soluble protein

• Southern blot analysis of HSA transgenic tobacco plants.

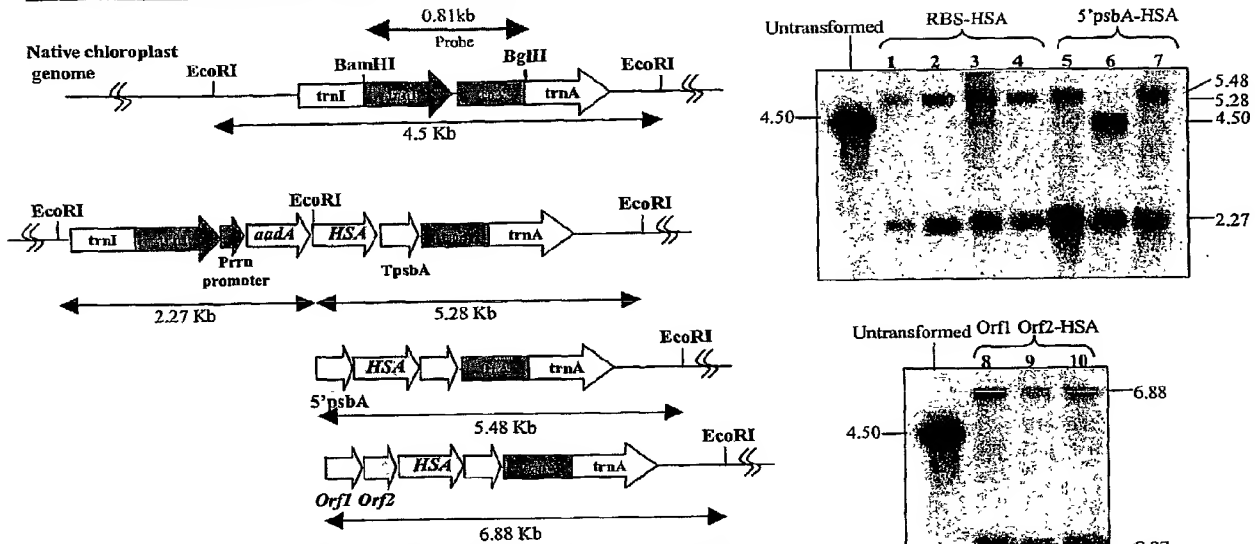


Figure 2: Southern Blot of HSA transgenic plants. Untransformed tobacco DNA vs. transgenic tobacco DNA digested with EcoRI. 1,2,3,4: DNA from plants transformed with pLD-RBS-HSA; 5,6,7: pLD-5'psbA-HSA; 8,9,10: pLD-Orf1-Orf2-HSA. Note homoplasmity in all the clones except number 6.

• Northern blot analysis of HSA transgenic tobacco plants.

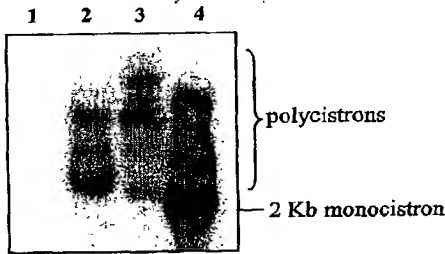


Figure 3: Northern Blot of HSA transgenic plants using HSA probe (1.8 kb). 1: untransformed tobacco RNA. 2: RNA from plants transformed with: pLD-RBS-HSA; 3: pLD-Orf1-Orf2-HSA; 4: pLD-5'psbA-HSA. Note different sizes of transcripts and the presence of monocistrons in number 4.

• ELISA analysis of HSA transgenic tobacco plants.

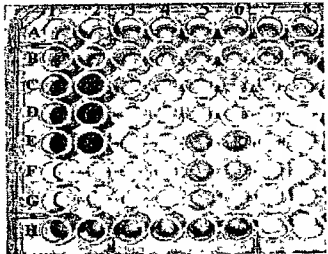


Figure 4: ELISA of HSA transgenic plants. A-E/1-2: HSA standards; F/1-2: Blank; G/1-2: Untransformed Petit Havana protein extracts; D-E/3-4: proteins from plants transformed with pLD-Orf1Orf2-HSA; F-G/3-4 and D-H/7-8: pLD-RBS-HSA; Rest of the wells contain extracts from different clones transformed with pLD-5'psbA-HSA.

• IGF-I optimized sequence and PCR product after synthesis of the new gene.

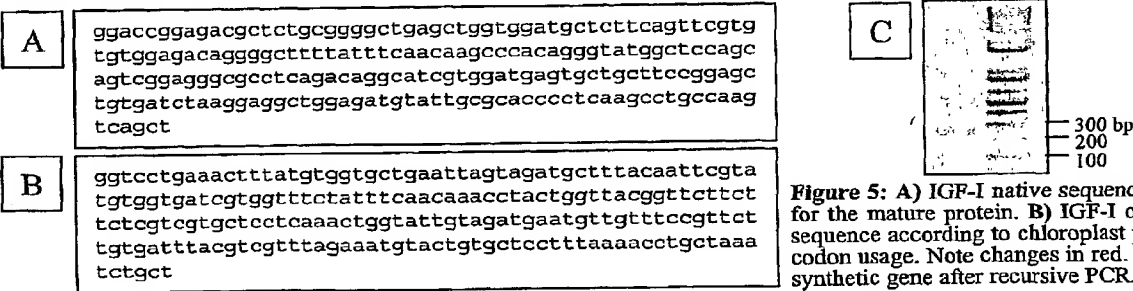
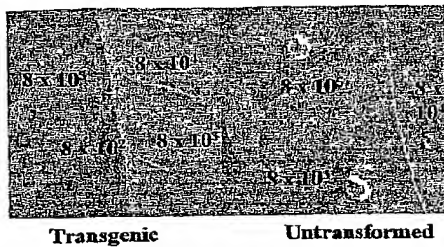
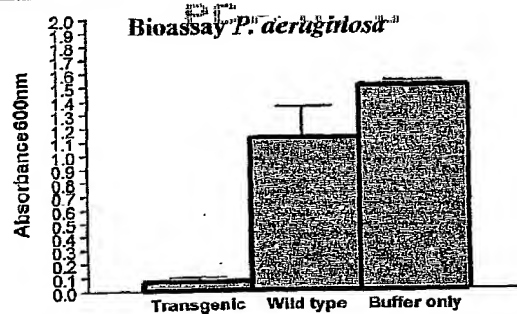


Figure 5: A) IGF-I native sequence coding for the mature protein. B) IGF-I optimized sequence according to chloroplast preferred codon usage. Note changes in red. C) IGF-I synthetic gene after recursive PCR.

• Expression of a small peptide in transgenic chloroplasts.

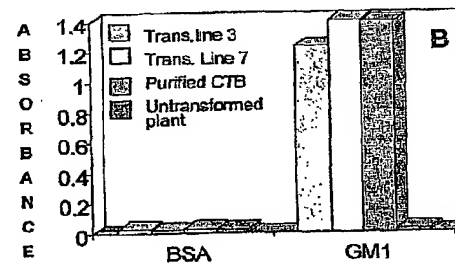
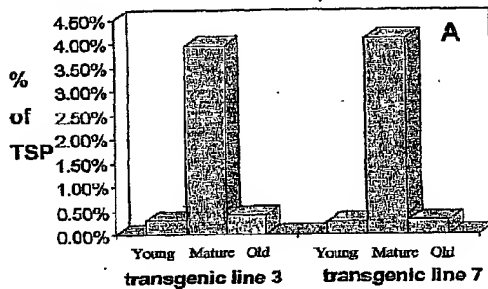


**Figure 1.** Leaves were infected with 10  $\mu$ l of  $8 \times 10^5$ ,  $8 \times 10^4$ ,  $8 \times 10^3$  and  $8 \times 10^2$  cells of *P. syringae*. Photos were taken 5 days after inoculation. 1-2  $\mu$ g of antimicrobial peptide (AMP) is required to kill 1000 bacterial cells. Local concentration at the site of infection is estimated to be 200-800  $\mu$ g AMP.



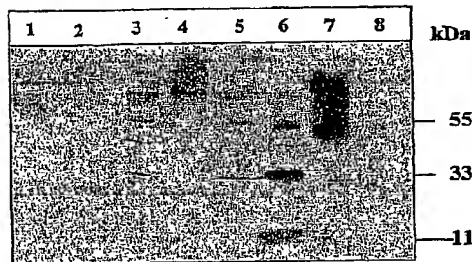
**Figure 2.** Total plant protein was mixed with 5  $\mu$ l of mid-log phase bacteria from overnight culture, incubated for 2 hours at 25°C at 125rpm and grown in LB broth overnight. Based on minimum inhibitory concentration of 1-2  $\mu$ g AMP/1000 bacterial cells, the expression level was calculated to be 21.5-43% of the total soluble protein.

• Expression of Oligomeric form (disulfide bonded) CTB in transgenic chloroplasts.



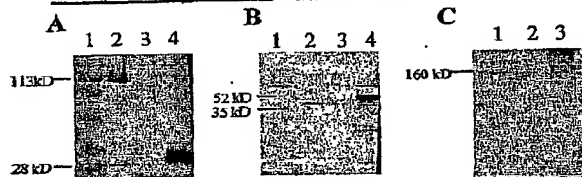
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• Expression of CTB oligomers.



**Figure 4:** 12% reducing PAGE. Chemiluminescent detection with rabbit anti-cholesterol serum (1<sup>st</sup>) and AP labeled mouse anti-rabbit IgG (2<sup>nd</sup>) antibodies. Untransformed, boiled (1) and unboiled (2); Transformed, boiled (3&5) and unboiled (4); Purified CTB boiled (6) and unboiled (7); Marker (8).

• Expression & assembly of disulfide bonded  
Guy's 13 monoclonal antibody.



**Figure 5:** A, B) reducing gels. 1: markers, 2: Transgenic extract showing expression of light (A) and heavy chain (B) in chloroplasts, 3: Untransformed, 4: Human IgA. C) non-reducing gel. 1: Transgenic extract showing assembly, 2: Untransformed, 3: Human IgA. Blots A & C were detected with AP conjugated goat anti-human kappa antibody. Blot B was detected with AP conjugated goat anti-human IgA antibody.

• Expression of HSA via nuclear genome in potato.

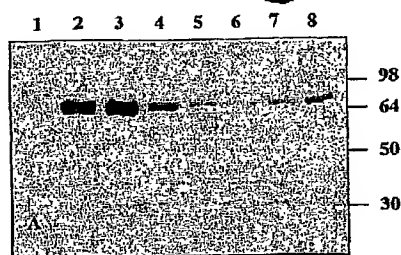


Figure 6: Western Blot of transgenic potato tubers, cv Désirée. 30 µg of tuber protein was loaded per lane and probed with anti-HSA antibody. 1: wild type; 2: 40 ng of pure HSA; 3-8: different transgenic lines, showing different levels of expression.

• Expression of HSA by chloroplast vectors in *E. coli*.

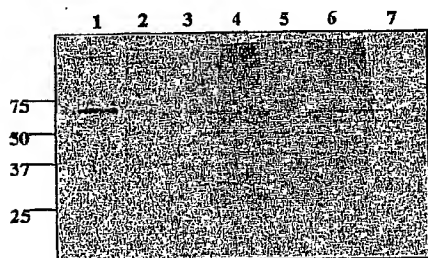


Figure 8: Western Blot of *E. coli* protein extracts. 1: 50 ng pure HSA; 2: molecular weight marker; 3: pLD-HSA (control without RBS); 4: pLD-5'UTR-HSA; 5: pLD-RBS-HSA; 6: pLD-ORF1+2-HSA; 7: *E. coli* without pLD vector.

• Expression of HSA via chloroplast genome in tobacco.

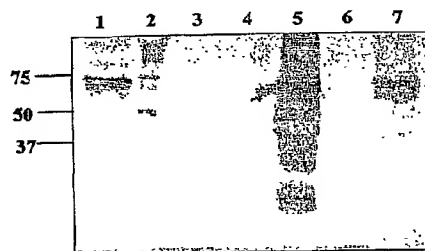


Figure 9: Western Blot of tobacco protein extracts. 1: 40 ng pure HSA; 2: molecular weight marker; 3 and 4: wild type plant extracts; 5: extracts from plants transformed with pLD-5'UTR-HSA; 6: pLD-RBS-HSA; 7: pLD-ORF1+2-HSA. 30 micrograms of plant protein were loaded per well.

• PCR analysis of transformants to determine integration of HSA gene into the chloroplast genome.

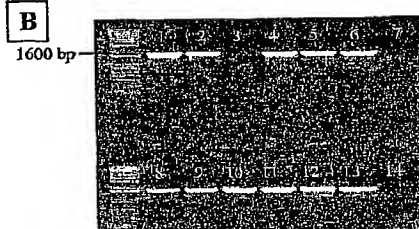
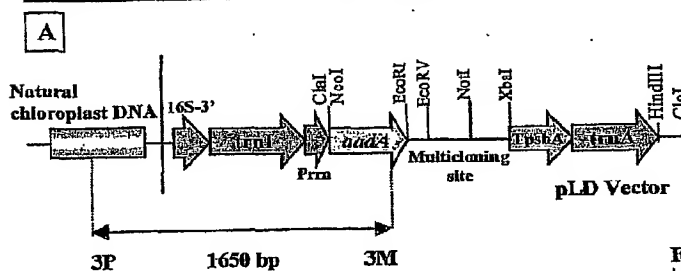


Figure 10: A) Map of the pLD chloroplast transformation vector and primer landing sites. B) Agarose gel containing PCR products using total plant DNA as template from plants transformed with: 1,2,3: pLD-RBS-HSA; 4,5,6: pLD-5'UTR-HSA; 8,9,10: pLD-ORF1+2-HSA; 11,12,13: pLD-ORF1+2-5'UTR-HSA; 7,14: negative controls (from untransformed plants); 3: mutant.